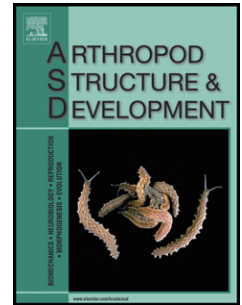


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Characterization of the cardiac ganglion in the crab *Neohelice granulata* and immunohistochemical evidence of GABA-like extrinsic regulation

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Abbreviations list:

CG cardiac ganglion

CIR cardiac inhibitory response

CNS Central nervous system

GABA Gamma-aminobutyric acid

GABAi GABA-like immunoreactivity

GT ganglionic trunk

HR heart rate

MMT modified Masson's trichrome staining

OCT optimal cutting temperature embedding

PB phosphate buffer

PBT Triton X-100 in PB

PI propidium iodide

SAL crustacean saline solution

ABSTRACT

The aim of the present work is to provide an anatomical description of the cardiac system in the crab *Neohelice granulata* and evidence of the presence of GABA by means of immunohistochemistry. The ganglionic trunk was found lying on the inner surface of the heart's dorsal wall. After dissection, this structure appeared as a Y-shaped figure with its major axis perpendicular to the major axis of the heart. Inside the cardiac ganglion, we identified four large neurons of $63.7 \mu\text{m} \pm 3.7$ in maximum diameter, which were similar to the motor neurons described in other decapods. All the GABA-like immunoreactivity (GABAi) was observed as processes entering mainly the ganglionic trunk and branching in slender varicose fibers, forming a network around the large neurons suggesting that GABAi processes contact them. Our findings strengthen previous results suggesting that the GABAergic system mediates the cardioinhibitory response upon sensory stimulation.

Keywords: cardiac inhibitory response; cardiac ganglion; extrinsic regulation; GABAergic system; decapods

1. INTRODUCTION

Sudden environmental stimuli of different modalities may either temporarily reduce (bradycardia) or interrupt the heart rhythm, inducing reversible cardiac arrests (Cuadras, 1981). Cardio-inhibitory responses (CIR) occur in many animal groups, such as mollusks (Wells, 1979; King and Adamo, 2006), crustaceans (Cuadras, 1980; Grober, 1990a; Burnovicz et al., 2009), fish (Axelsson et al., 1987; Axelsson et al., 1989; Ide and Hoffmann, 2002), amphibians (Laming and Austin, 1981), birds (Cohen and Macdonald, 1971) and mammals (Smith et al., 1981). Several studies have attempted to explain a possible functional role of this transient cardiac inhibition in response to external stimulation. CIR has been associated both with attentional phenomena in vertebrates (Powell, 1994) and with the orienting response in invertebrates (Shuranova and Burmistrov, 1996). In addition, CIR has been described as a component of the “death-feigning behavior” in decapod crustaceans (McMahon and Wilkens, 1972) and as an index of sensory perception and evidence of a neuroautonomic response in the crab *Neohelice granulata* (Hermitte and Maldonado, 2006; Burnovicz et al., 2009; Burnovicz and Hermitte, 2010). In any case, its prevalence across invertebrates and vertebrates leads to the assumption that it has a universally adaptive function. However, the underlying mechanisms of CIR are still unknown.

The basic contraction rhythm of the decapod crustacean heart emerges from the bursting discharges of the cardiac ganglion (CG), a model of pacemaking and central pattern generation (Welsh and Maynard, 1951; Wiens, 1982; Marder and Calabrese, 1996; Cooke, 2002). The CG, which consists of the neurons and their processes, glia and connective tissue, forms an

elongated, discrete branching trunk either in or on the heart. In most of the decapods examined nine neurons can be found with a clear distinction in size, function and localization (Cooke, 2002).

Complex modulation by CNS inputs and by neurohormones to adjust heart beating to physiological needs has long been reported. The beating of the neurogenic heart is regulated by extrinsic nerves arising from the CNS (Alexandrowicz, 1932). In intact and semi-intact heart preparations, stimulation of the excitatory nerves speeds the contraction rate, and stimulation of the inhibitory nerves either slows down or stops the heart (Maynard, 1953; Florey, 1960; Watanabe et al., 1968; Watanabe et al., 1969; Ando and Kuwasawa, 2004). *En passant* recordings from the dorsal inhibitory nerves in semi-intact animals have revealed periodic increases in inhibitory nerve firing rates correlated with bradycardia (Field and Larimer, 1975a; Young, 1978). The activity of these nerves has also been characterized during sensory-induced modification of the heart rate (HR), and three sensory modalities (stretch, chemical and tactile) have been found to mediate inhibitory cardiac reflexes (Field and Larimer, 1975a). In the lobster *Homarus americanus*, the successful cutting of the dorsal nerves was confirmed by the loss of the brief period of bradycardia known as startle response usually triggered by sensory stimulation (Guirguis and Wilkens, 1995).

Although crustacean cardiac ganglion neurons have been shown to respond to a number of neurotransmitters and modulators (Cooke and Hartline, 1975; Miller et al., 1984; Saver et al., 1999; Cruz-Bermudez and Marder, 2007), the identity of the neurotransmitters involved in most of the chemical synapses of the cardiac system remains uncertain. However, the

innervations of the ganglion motor neurons by extrinsic cardio regulatory fibers has been extensively examined and considerable evidence indicates that GABA mediates inhibition at these synapses in isopods (Tanaka et al., 1992) and stomatopods (Ando and Kuwasawa, 2004). In decapods, the application of either GABA or the GABA_A agonist muscimol mimics the effects of the cardio regulatory fibers (Maynard, 1961; Yazawa and Kuwasawa, 1992, 1994). Both actions are blocked by specific antagonists such as picrotoxin (Shimahara, 1969a; Ando and Kuwasawa, 2004), and both cause an enhancement in the conductance of Cl⁻ in the motor neurons (Shimahara, 1969a; Kerrison and Freschi, 1992). More recently, a study of GABA-like immunoreactivity (GABA_i) in the CG of *Panulirus argus* (Delgado et al., 2000) showed that all the GABA_i in the CG originates from a single bilateral pair of fibers that enter the heart via two dorsal nerves and forms extensive processes in the neuropil throughout the ganglion, including a pericellular network around large cell somata. Processes have also been seen to ramify into the heart together with the large-cell dendrites presumed to be mediating stretch sensitivity. This morphology strikingly matches the description by Alexandrowicz (1932), who early proposed that the System I fibers were inhibitory.

The aim of this investigation was to study the anatomy of the inhibitory input to the heart that may mediate the CIR. Until the present study, a macroscopic and cellular description of the anatomy of the CG of *Neohelice granulata* and the principal neurotransmitters involved in the extrinsic control of CIR had not been reported. Thus, we aimed to provide: a) an anatomical description of the cardiac system of this species based on classical histological staining techniques, characterizing the cardiac ganglionic trunk (GT) and its components, and b) evidence of the presence of GABA by means of immunohistochemistry.

Unpublished data from our own experiments suggest that rapid CIR to environmental disturbances, reminiscent of an autonomic-like regulation, may be extrinsically regulated by the GABAergic system. The presence of GABA_i in the cardiac system strongly implicates that this neurotransmitter might be an extrinsic mediator of CIR behaviors.

2. MATERIALS AND METHODS

2.1. Animals

Animals were adult male *Neohelice granulata* (previously *Chasmagnathus granulatus*, Crustacea, Grapsidae, Dana, 1851) crabs 2.7–3.0 cm across the carapace, weighing around 17.0 g, collected from water less than 1 m deep in the “rías” (narrow coastal inlets) of San Clemente del Tuyú, Argentina (36° 21 ' S, 56° 43 ' W), and transported to the laboratory, where they were lodged in collective tanks (350 x 480 x 270 mm) of 20 animals each. The water used in the tanks was artificial sea water prepared with *Red Sea Salt* (Red Sea Fish Pharm. Ltd.), salinity 1.0 – 1.4‰ and pH 7.4 – 7.6. The holding and experimental rooms were kept on a 12-h light/dark cycle (light on 07:00–19:00 h). Experimental procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (USA), and the Argentinean guidelines on the ethical use of animals.

2.2. *In vivo* latex vascular casting

We performed *in vivo* latex vascular casting to investigate the anatomy and morphology of the heart and vascular system of *Neohelice*. Colored fluid latex is widely used in human medical anatomical research of the circulatory and respiratory systems because it maintains the flexibility and plasticity of the tissues at a level equivalent to fresh material. The vascular filling with latex is carried out without difficulty and allows reaching the smallest vessels (<1mm). In crustaceans, latex perfusion has been used to trace the course of vessels of the cardiac system (Steinacker, 1978; Gribble, 1994). We used fluid pre-vulcanized latex (Latex Co. SACIF, Buenos Aires, Argentina), which is easy to handle, liquid at room temperature, and solidifies rapidly by dehydration. To prevent hemolymph loss, only the dorsal carapace over the cardiac region was removed. Pure latex or latex diluted 1:1 or 1:3 in crustacean saline solution (SAL) (Hoeger and Florey, 1989) was injected right into the heart using a micro-capillary or a 1-ml syringe. When crabs were anesthetized in chilled water, the heart rate was reduced. However, it still showed a robust and sustained activity for several minutes, which allowed for latex circulation and distribution.

2.3. Heart isolation procedure

Crabs were anesthetized in chilled artificial seawater before dissection. The heart was accessed by removing the overlying carapace and connective tissues. The alary ligaments, which suspend the heart in the pericardial cavity, were cut and the heart was removed. Finally, the isolated heart was pinned ventral side up in a silicone elastomer dish (Sylgard) using entomological needles and the ventral wall was cut open to expose the CG. The isolated heart was bathed in SAL and the CG was exposed using a pair of fine dissecting forceps.

To aid neuronal and nerve visualization, some preparations (n=30) were stained with methylene blue and propidium iodide.

2.4. Histological techniques

2.4.1. Methylene blue staining protocol

Hearts were isolated and ganglia were exposed as outlined above and stained by means of a vital staining technique, methylene blue. Reduced methylene blue solution was prepared from 0.5% methylene blue in 0.1 M phosphate buffer (PB 0.1 M: 0.2 M NaH_2PO_4 , 0.2 M Na_2HPO_4 , pH 7.4) and titrated with a 500 mM dithiothreitol solution (Sigma-Aldrich) until colorless. A drop of the reduced colorless solution (50 μl) was poured over the exposed ganglion. The preparations were observed under a low-power dissection microscope (SZX7, Olympus) and under a microscope (CX-31, Olympus) at magnifications of 4x/NA=0.1, and 10x/NA=0.25, 40x/NA=0.65 and photographed with a digital camera (Infinity2-1, Lumenera Corporation, Ottawa, ON, Canada).

2.4.2. Propidium iodide staining protocol

Hearts were isolated and ganglia were exposed as outlined above, fixed in 4% paraformaldehyde in PB for 30 min at room temperature, washed twice with 1% non-ionic detergent Triton X-100 in PB (PBT) for 10 min and a drop (50 μl) of 1.5 μM propidium iodide solution (PI, Molecular Probes, Cat. No. P-130) was added to the preparation, incubated for 15

min and washed twice with PB. Specimens were analyzed and scanned as described in section 2.8.

2.4.3. Modified Masson's trichrome staining protocol

Specimens were fixed in Bouin solution (prepared with 70% of saturated picric acid solution, 25% of formaldehyde at 4% solution and 5% of acetic acid) for 1h at room temperature, dehydrated in a graded ethanol series (70, 90, 96 and 100%) and cleared in xylene. Specimens were embedded in a paraffin (Histoplast®, Biopack, Argentina)-xylene 50%-50% solution for 30 min at 58°C, and then moved into individual containers for 6h at 58°C. Paraffin blocks were serially sectioned at 25µm using a microtome (model 1345, Leitz Wetzlar, Germany; blades from Leica, model 818, Leica Instruments GmbH). Sections were taken from flotation onto glass slides previously agar-treated or electrostatically charged (Superfrost® Plus, Thermo Scientific). The slides were dried (10 min at 60°C), deparaffinized, rehydrated in a graded ethanol series (96, 70 and 50%) and immersed in distilled water.

For Modified Masson's trichrome staining (MMT), slides were immersed in Carazzi's hematoxylin (prepared from 0.5g Hematoxilin from Sigma-Aldrich Cat. No. H-9627, 25g aluminum potassium sulphate, 0.1g potassium iodate, dissolved in 200ml of distilled water and 100ml of glycerol) for 10 min, washed in tap water, immersed in acid fuchsin xylidine ponceau for 5 min and then washed in distilled water. Next, slides were first immersed in 1% phosphomolybdic acid solution for 5 min and then dipped in acetic aniline blue for 3 min without washing. Hematoxylin is expected to stain cellular nuclei in violet, while fuchsin

xylydine ponceau stains cytoplasm in pink. Aniline blue stains mucus and cellular matrix in light blue and collagen fibers in blue.

After staining, all preparations were dehydrated in ethanol 96%, dried at 37°C, cleared in xylene and mounted in synthetic permanent mounting medium (PMMyR, Instrumental Pasteur, Buenos Aires). The preparations were observed under a microscope (CX-31, Olympus) at magnifications of 4x/NA=0.1, 10x/ NA=0.25, 40x /NA=0.65 and 100x/ NA=1.25 and photographed with a digital camera (Infinity2-1, Lumenera Corporation, Ottawa, ON, Canada). Pictures were saved as .BMP files and analyzed with Image J (Image J 1.44c, NIH, USA) for scaling, measuring, cropping, labeling, and adjustment of overall brightness and contrast. Maximum cell diameter was determined individually in stacked images, selecting the slide of maximum cell equator.

2.5. Embeddings for GABA immunohistochemistry

Specimens were fixed in 4% paraformaldehyde in PB for 2h at room temperature, then washed twice in PB for 10 min. After fixation, specimens were included in paraffin, optimal cutting temperature (OCT) or agarose. The paraffin embedding procedures are described above in section 2.4.3.

2.5.1 Agarose embedding

After fixation and washing, specimens were dried with absorbent paper. Hearts were placed in individual embedding molds (E-4265, Sigma-Aldrich) filled with 7% low melting point agarose (Promega, Madison, WI, USA) previously heated to 70°C. Agarose blocks were serially sectioned at 200 μm with a vibratome (Series 1000 sectioning system, Vibratome; blades from Schick Super Chromium®, Schick, Germany).

2.5.2 Optimal Cutting Temperature embedding

After fixation and washing, specimens were immersed for cryoprotection in increasing sucrose solutions of 5, 10, 15 and 20%, for 20 min each. After drying in absorbent paper, hearts were placed in individual embedding molds filled with OCT (Cryoplast®, Biopack Argentina). The OCT blocks containing the hearts were cooled in liquid nitrogen for 2 min and serially sectioned at 25 μm with a cryomicrotome at -20°C (Cryocut-E, Reichert Jung, Germany; blades from Reichert Jung, Germany). Sections were taken from flotation onto glass slides previously agar-treated or electrostatically charged (Superfrost® Plus, Thermo Scientific).

2.6. GABA immunohistochemistry

Polyclonal anti-GABA antibody (raised in rabbit, Sigma-Aldrich, Cat. No. A-2052) was used to detect GABA-like immunoreactivity (GABA_i). Specimens were preincubated with bovine serum albumin (BSA, 2% in PB, for 2 h at room temperature) and washed twice for 10 min in 0.5% PBT. Sections were immersed in the anti-GABA primary antibody raised in rabbit, diluted 1:500 in PB and 0.3% Tween, incubated overnight at 4°C with agitation and then

washed in PBT (x3, for 10 min). Following incubation in the secondary antibody in a dilution 1:500 (anti-rabbit raised in goat and conjugated to Alexa 488 (Invitrogen, Life Technologies, Cat. No. A-11008) for 2–4 hours at room temperature, specimens were washed for 10 min in PBT and for 10 min in PB alone. Agarose-embedded thick sections were immersed in a PB-glycerol solution (50:50) for 30 min, and then mounted on glass slides with a PB-glycerol solution (20:80) and 1.5 μ M PI. Paraffin or OCT thin sections were first stained with PI for 10 min, then dehydrated in a graded ethanol series, cleared in xylene and mounted in methyl salicylate medium. PI binds to DNA and RNA by intercalating between the bases, staining the nucleus as well as the cytoplasm. When bound to nucleic acid, the fluorescence maximum emission of PI is 617 nm. PI was used as a counter-staining for fluorescence immunochemistry and cellular morphology.

2.7. Control experiments

For negative controls, an anti-GABA antibody was preabsorbed with GABA crosslinked to BSA (Sigma-Aldrich, Cat. No. A-4503). The protocol followed the method of Meyer (Meyer et al., 1991): 30mg of BSA, 50 mg of GABA and 100 μ l of 25% aqueous glutaraldehyde were dissolved in 2ml of PB. After 20 h incubation, GABA –BSA were separated from low molecular weight material on a Sephadex G-25 column. In order to preabsorb the antibody, 0.6 μ g of antibody was incubated overnight with 20 μ g of GABA-BSA at 4°C with agitation.

Another control was performed incubating the sections with normal rabbit serum in a dilution 1:500 (normal rabbit IgG; Santa Cruz Biotechnology, sc-2027) overnight at 4°C with agitation.

We did not perform specificity tests for our anti-GABA antibody (Sigma-Aldrich, Cat. No. A-2052) taking into account that it had already been done (Yang et al., 1997). These authors assessed the specificity of the same antibody, using a variety of amino acids (alanine, aspartate, glutamate, glutamine, glycine, taurine) and amines (dopamine, noradrenaline, 5-hydroxytryptamine) conjugated with glutaraldehyde to BSA and immobilised on nitro-cellulose (Hodgson et al., 1985). The antibody did not show a cross-reaction with conjugates.

2.8 Analysis of fluorescence

Fluorescence signals were analyzed and scanned using an argon 488 laser confocal microscopy (Olympus FV300, 488 nm for Alexa 488 and PI) and appropriate emission filters (BA510IF and BA530RIF for channel 1 and 605BP (575-630) for channel 2). Optical sections were spaced 3 μm apart and magnifications were of 10x/ NA=0.30, 20x /NA=0.5 and 60x/ NA=1.4. Pictures were saved as .TIFF files and analyzed using Image J (Image J 1.44c, NIH, USA) with FVControl plug-in for detailed image analysis, scaling, measuring, cropping, labeling, and adjustment of overall brightness and contrast.

Maximum cell diameter was determined individually in stacked images, selecting the slide of maximum cell equator.

3. RESULTS

3.1. Cardiac system anatomy

The anatomy of the cardiac system of *Neohelice* had not been described until the present work. The cardiac region, which can be identified in the dorsal surface of the carapace, is situated immediately above the heart (Fig. 1A). After carapace removal, the heart was observed in a dorsal position inside the pericardial cavity, which is located in the posterior region of the cephalothorax and flanked by two septa that separate it from the gills (Fig. 1B). The heart has a rectangular shape and an approximate size of 5.50mm (major axis) x 1.75mm (minor axis) and was visible with the naked eye. Vigorous heart beating was observable directly or under low-power dissection microscope.

Latex injected *in vivo* spread easily throughout the circulatory system, allowing us to observe the morphology and to identify the main dorsal and ventral vessels. The dorsal hepatic and lateral arteries emerged from the anterior region of the heart, supplying the hepatopancreas and guts respectively (Fig. 1C). The conspicuous sternal artery exited from the ventral surface of the heart and, after passing through the foramen of the thoracic ganglion (Fig. 1D), headed to the ventral region of the body, where it divided into thinner branches, distributing the hemolymph to the mouthpieces, chelae and the four pairs of limbs (Fig. 1E).

3.2. *In situ* characterization of the cardiac ganglion

After removal of the carapace over the pericardial region, the CG of *Neohelice* was seldom discernible with the naked eye through the transparent cardiac muscle. However, after cutting open the ventral wall of the heart, the GT appeared as a white and opaque Y-shaped structure, 0.8-1.0 mm in length, located in the inner surface of the dorsal wall and partially entangled

with muscle fibers (Fig. 1F). This location and general aspect was in agreement with numerous previous descriptions of the CG in crustaceans (Alexandrowicz, 1932; Maynard, 1953; Yazawa and Kuwasawa, 1994; Saver et al., 1999; Cooke, 2002).

After the foundational work of Alexandrowicz (1932), several other authors have used vital methylene blue staining to study crustacean heart innervation as it is well known that it stains elements from the nervous system (Maynard, 1953; Sakurai et al., 1998; Saver et al., 1999; Delgado et al., 2000; Ando and Kuwasawa, 2004). Our results with methylene blue allowed us to trace the cardiorespiratory nerves, presumably the pair of conspicuous nerves that enter laterally and run directly into the anterior region of the GT at the level of the three large ganglion cells (Fig. 2A).

We used both methylene blue and PI staining techniques to determine the number of neurons in the CG of *Neohelice*. After staining several ganglia ($n=30$), we were able to pinpoint the area in which they resided. The entire ganglion was surrounded by a thick connective tissue sheath, which was either removed with fine forceps to aid neuronal visualization or permeabilized to aid dye penetration. In more than 25 isolated heart preparations, we counted four large neurons in the area of the CG: three of them in the anterior part of the trunk, where several blue-stained branches converge, and the other in the thinner part of the GT, heading posteriorly (Fig. 2A, B).

The MMT staining technique was used to describe the general anatomy of the CG of *Neohelice* in more detail. About 30 to 40 sections of 25 μm thick, were obtained from each paraffin-embedded heart. The CG was neither observed in the first nor in the last sections, confirming

its location in the inner surface of the dorsal wall as in other decapods (Alexandrowicz, 1932; Saver et al., 1999; Cooke, 2002). Additionally, taking into account that the CG appears in approximately 4-6 consecutive paraffin sections, we estimated the whole structure was 100-150 μm thick. We repetitively observed the presence of large neurons and neighboring nuclei from smaller cells enclosed in a pyriform and discrete structure wrapped in connective tissue, localized in the central region of the heart. Its approximate size was $450\mu\text{m} \times 130\mu\text{m}$, positioned with its major axis perpendicular to the major axis of the heart (Fig. 2 C).

The four large neurons located within the GT had $63.7 \pm 3.7\mu\text{m}$ (mean \pm S.E.M., $n=15$) in maximum diameter (Figs. 2A, B, D and supplementary material in Fig. 5). The cytoplasm of these cells presents outstanding basophilia and granulous aspect, as well as a clear, large and oval nucleus with a conspicuous nucleolus. There were also abundant nuclei of $8.63 \pm 1.07\mu\text{m}$ (mean \pm S.E.M; $n=225$) in maximum diameter corresponding to smaller sized cells surrounding the large neurons (Fig. 2E).

3.3. GABA-like immunoreactivity

Since the morphology of the heart is dorsoventrally asymmetric, it was relatively easy to establish the spatial position of a structure in relation to the section being cut. On the outer dorsal surface, two conspicuous pouches protruded, whereas on the ventral surface, sections showed a more uniform morphology of the heart (Scheme in Fig. 3A).

Coronal OCT sections of the heart showed GABAi in the central region of the GT as a Y-shaped figure located centrally (Fig. 3A). GABAi processes seemed to originate from fibers that enter the heart laterally from the sides reaching the GT region. Upon entering the GT, the GABAi axons gave rise to extensive arborizations within the CG, as observed in greater magnification (Fig. 3B). The CG and nerves appeared wrapped in connective tissue.

In a sagittal agarose section, GABAi processes were observed running along the anterior-posterior axis of the GT (Fig. 3C). Figure 3C also shows that the CG is strongly attached to muscular tissue of the dorsal wall of the heart and exposed to its lumen.

The CG region also presented two long GABAi processes of 10 μ m of maximum diameter surrounding the large cells described above. These fibers gave rise to varicosities forming a network around the cells (Figs. 3 D, E).

No immunoreactivity was detected in control preparations (Figs. 3F, G). Both the preabsorption of the anti-GABA antibody with GABA-BSA and the incubation of the tissue with normal rabbit IgG resulted in complete absence of GABAi. The CG was visible only through the PI staining together with some autofluorescence corresponding to muscle tissue. This strongly suggests that the GABAi observed was specific.

3.4 General organization of the ganglionic trunk in *Neohelice*.

Figure 4 is a drawing composite from several independent observations of fresh and methylene blue stained heart preparations in which we describe the general organization of the GT, number, location, and position of the CG neurons as well as the general shape, position and size of the GT. Its Y-shaped structure, of approximately 0.8 -1mm in length, spanned the heart along the midline. The main parts of the local system could be observed lying on the inner surface of the dorsal wall. This local system consisted of a trunk containing large neurons and branches arising from it and distributed in the heart muscles. The two branches originating from the anterior bifurcation headed laterally, turned backwards, and took a circular course until they reached the posterior end of the GT. In this way, they presumably put the anterior and posterior part of the median trunk into communication. From the circular anastomosing branches, thinner nerves to different parts of the heart ramified diagonally. Presumably, the dorsal nerves entered the heart from its dorsal side and joined the local system laterally, reaching the central region where the three large cells were observed.

4. DISCUSSION

The purpose of this investigation was to study the anatomy of inhibitory input to the heart that might mediate the extrinsic control of cardio inhibitory responses. With this aim, we examined the cardiac system of *Neohelice granulata* and described the general morphological and organizational aspects of the GT and CG by classical histological staining techniques, and searched for evidence of GABAergic extrinsic regulation in the CG region by immunohistochemistry. This latter finding strengthened unpublished data from our own experiments, suggesting that the extrinsic inhibitory regulation of the cardiac response may be

mediated by GABA as Picrotoxin, a non competitive GABAergic antagonist, partially abolished the CIR already described in this decapod (Hermitte and Maldonado, 2006; Burnovicz et al., 2009; Burnovicz and Hermitte, 2010).

Three basic elements were early described in the decapod cardiac system: a) a group of neurons situated in the heart itself and therefore constituting its local system, b) fibers which connect this local system with the CNS and are represented by the dorsal nerves and c) a system of fibers innervating the cardiac muscle and valves situated at the exit points of the arteries (Alexandrowicz, 1932). Those equivalent constitutive elements found in *Neohelice* are discussed below.

4.1. The cardiac ganglion: the local system

The nerve cells which, together with their processes, build up the local system. In the crustacean CGs, a discrete number of ganglion cells (between 6 and 16) can be found, with a clear distinction in size, function and localization. In most of the decapods examined, the number is lower (9). The more posterior ones are smaller and having axonal terminations within the neuropil in the ganglionic trunk are considered interneurons. In contrast, the more anterior ones are larger and, because they provide axons that abandon the ganglion to innervate muscle fibers, they are regarded as motor neurons (Delgado et al., 2000; Cooke, 2002; Ando and Kuwasawa, 2004; Fort et al., 2004). In more than 25 heart preparations, we counted four large neurons, which due to their size ($63.73 \pm 3.74 \mu\text{m}$ mean \pm S.E.M) in maximum diameter and location, were considered putative motor neurons and equivalent to those described in other decapod species.

The distribution of the neurons inside the GT seems to depend on the size and shape of the animal. In lobsters, the somata are widely spaced along a linear (e.g. *Panulirus*) or Y-shaped (e.g. *Homarus*) trunk which is relatively shorter in Brachyura. In a frequent arrangement in crabs, the neurons are usually compacted into anterior and posterior clusters separated by several millimeters of GT (Tazaki and Cooke, 1983; Cooke, 2002). This segregated distribution has also been found in the smaller size decapod species, where the small cells were difficult to locate in most preparations. Moreover, the small cells were frequently encased in a “pocket” of connective tissue buried deep within the main trunk of the CG at its distal end (Saver et al., 1999). This basic plan fits with the distribution observed in *Neohelice*. Three large neurons where the GT is relatively widened in the anterior part and a fourth large cell located more posteriorly in the thinner portion of the GT were always observed. However, we could not identify the small neurons in their supposed location associated with the large cell located more posteriorly in the GT or at the distal end.

4.2. General organization of the ganglionic trunk in *Neohelice*.

We are proposing a general organization of the GT in *Neohelice* as a line drawing composite from several methylene blue staining and isolated heart preparations (Fig. 4 and supplementary material in Fig. 5). Here we describe the number, location, and position of the CG neurons as well as the general shape, position and size of the GT which was found comparable to that described in other decapods (Alexandrowicz, 1932; Maynard, 1953; Cooke, 1962; Saver *et al.*, 1999; Cooke, 2002)

4.3. Evidence of GABA-like immunoreactivity in the CG region in *Neohelice*

The electrophysiological and anatomical studies of Maynard (1953) of the CNS of another decapod, the spiny lobster *Panulirus argus*, established that a bilateral pair of large diameter cardio-inhibitory fibers originated in the thoracic region and reached the CG entering dorsolaterally. Our results with methylene blue allowed us to trace the cardioregulatory nerves, presumably the pair of conspicuous nerves that enter laterally and run directly into the anterior region of the GT at the level of the three large ganglion cells. Immunohistochemical preparations showed GABA_i distributed in the heart in a Y shaped fashion suggesting that GABA_i processes enter the heart and reach the CG laterally from both sides.

Within the CG, these GABA_i processes gave rise to extensive varicosities that entangle around the somata of the large neurons forming perisomatic networks. The GABA_i in the CG of *Neohelice* showed localization of terminals in close proximity to the large cell somata, suggesting axo-somatic contacts. However, terminals were also observed distant from the cell somata, suggesting possible axo-dendritic contacts. This distribution resembles previously described GABAergic immunoreactivity in the GC of other decapods (Delgado et al., 2000).

5. ACKNOWLEDGEMENTS

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Characterization of the cardiac ganglion in the crab *Neohelice granulata* and immunohistochemical evidence of GABA-like extrinsic regulation

Authors: Margarita Yang, Martín Carbó Tano, Ramiro Freudenthal and Gabriela Hermitte

Highlights

- The gross morphology of the cardiac system and the ganglionic trunk is described.
- Four large ganglion cells have been identified and characterized within the cardiac ganglion.
- GABA-like immunoreactive processes were found surrounding the large neurons.
- These processes might mediate extrinsic regulation during cardioinhibitory responses.

LEGENDS

Figure 1. Morphology of the cardiac system and cardiac ganglion of *Neohelice*.

A. A drawing of the dorsal view of a *Neohelice* adult male. The cardiac region (CR) is located centrally and posteriorly on the carapace.

B. A drawing of the dorsal view after carapace removal. The heart (H) can be observed within the pericardial cavity, which is located in the posterior region of the cephalothorax. The heart is anchored by alary ligaments to a septum (S) on each side and flanked by the gills (G).

C. A close-up view of the pericardial cavity. The latex vascular cast of the heart (H) of *Neohelice* reveals its shape. The hepatic artery (HA) and lateral artery (LA) emerge from the anterior region of the heart.

D. Vascular cast of the conspicuous sternal artery (SA) after heart removal. The SA passes through the foramen of the thoracic ganglion (TG, dashed line) heading to the ventral region of the body.

E. A ventral view of a vascular cast of the sternal artery (SA). After passing through the foramen of the TG, the SA divides into thinner branches which reach the mouthpieces, chelae and the four pair of limbs.

F. Location and gross morphology of the ganglionic trunk (GT) in *Neohelice*. Inset: A scheme showing the overall shape of an isolated heart as well as the location of the four ostia. A magnification of the dashed line area shows the GT partially entangled within cardiac muscle fibers (MF) as observed immediately after dissection and without staining. The GT appears as a Y-shaped structure, giving off two branches which head laterally and a third one heading to the posterior region. Additionally, a thinner one heads anteriorly.

Figure 2. Characterization of the cardiac ganglion by staining techniques.

A, B. An overall view of the Y-shaped GT stained with methylene blue (A) and with propidium iodide (B). Three large neurons can be observed grouped in the anterior region of the CG, while a fourth one can be seen more posteriorly (arrow heads). Two nerves, presumably dorsal nerves (DN) which enter laterally from each side, are partially entangled within the cardiac muscle fibers (MF) and run directly into the anterior region where the large neurons are located. Thinner nerves to different parts of the heart ramified diagonally from the CG (Diag. N in panel B).

C. Localization of the CG in a longitudinal paraffin section of the heart of *Neohelice* stained with Modified Mason's Trichrome (MMT) staining. An elongated and discrete pyriform structure (dashed circle) can be observed in the central region, positioned with its major axis perpendicular to the major axis of the heart. Inset: In a higher

magnification, a group of three large neurons can be observed surrounded by nuclei from smaller cells, all wrapped in a sheath of connective tissue (arrow head).

D. A view in higher magnification of a CG stained with PI. Three large neurons are grouped anteriorly (Di), while a fourth one stands individually more posteriorly (Dii). The insets show a detailed view of the neurons.

E. The aspect of a large ganglionic neuron (arrow head) inside the CG as observed in a MMT-stained preparation. The neuron has a soma of $63.7 \pm 3.7 \mu\text{m}$ (mean \pm S.E.M., $n=15$) in maximum diameter (MD_1), granular cytoplasm and a clear basophile nucleus with a relaxed state of chromatin and a conspicuous nucleolus (arrow). Abundant nuclei of $8.63 \pm 1.07 \mu\text{m}$ (mean \pm S.E.M; $n=225$) in maximum diameter (MD_2) corresponding to smaller sized cells can be seen surrounding the large neuron.

Figures B and D are maximum intensity projection along the z axis. B, stack of 25 images. D, stack of 22 images, pixels were inverted and contrasted.

Figure 3. Evidence of GABA-like immunoreactivity within the ganglionic trunk and cardiac ganglion of *Neohelice*.

A. Inset: a coronal plane section of the heart.

In a central coronal agarose-embedded section of the heart, GABA_i is observed as a Y-shaped figure (dashed outline).

B. Higher magnification of panel A, GABAi processes give rise to extensive arborizations which span along the GT. Connective sheath indicated with arrow head.

C. Inset: a sagittal plane section of the heart.

In a central sagittal agarose section of the heart, GABAi processes run along the anterior-posterior axis of the GT (dashed outline). These processes give rise to varicose fibers that extend along the CG, which appears attached to the dorsal wall of the heart and exposed to its lumen (L).

D-G. GABAi (green), PI (red). **D,E.** Anterior region of the CG where large neurons can be observed (arrow heads). A GABAi process of 10 μm in diameter (arrow) gives rise to extensive varicosities forming perisomatic network in close proximity with the large neurons somata (arrow heads).

Control experiments: **F.** Incubation with normal rabbit IgG and **G.** Preabsorption of the anti-GABA antibody with GABA-BSA. No immunoreactivity was detected. Arrow heads in each panel indicate large neuronal somata stained with PI.

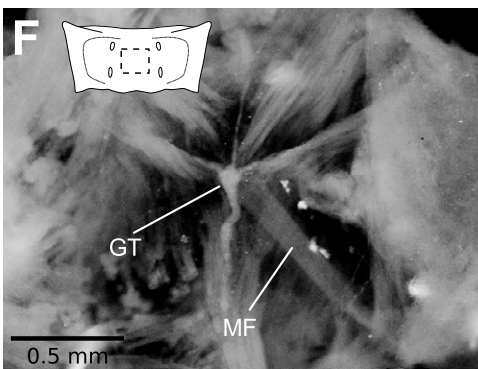
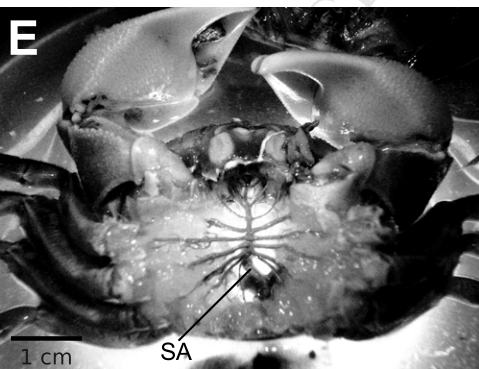
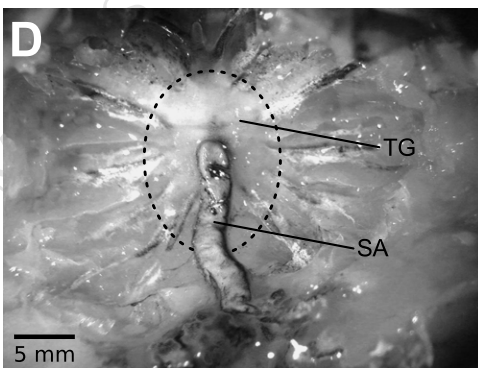
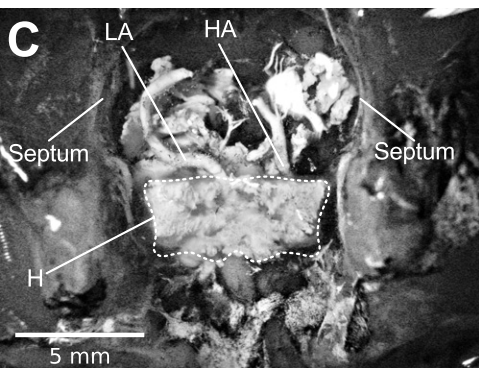
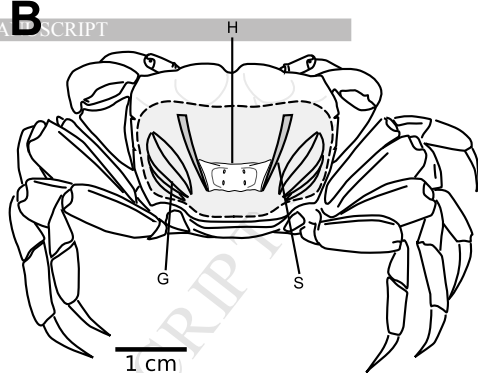
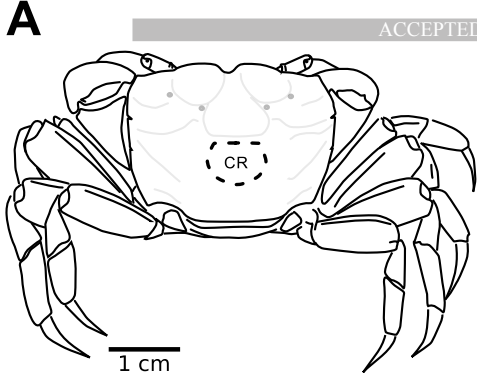
Figures A-G are maximum intensity projection along the z axis. In A-C) pixels were inverted and contrasted. A, stack of 31 images. B, stack of 31 images, C stack of 6 images, D stack of 8 images and E stack of 6 images.

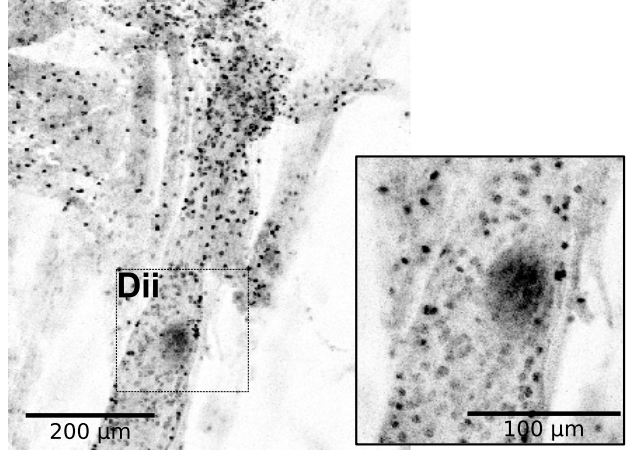
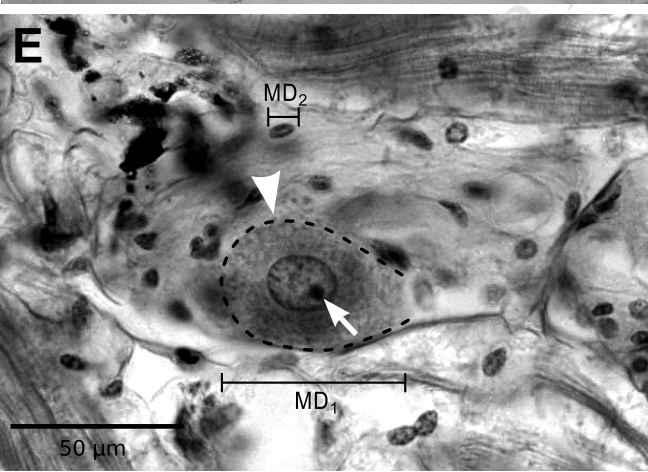
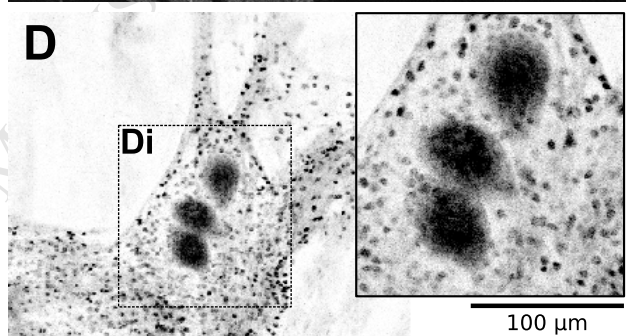
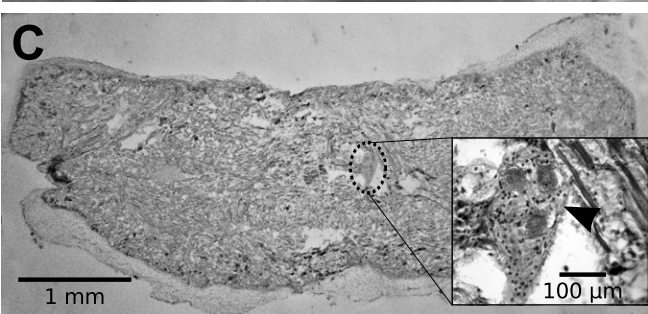
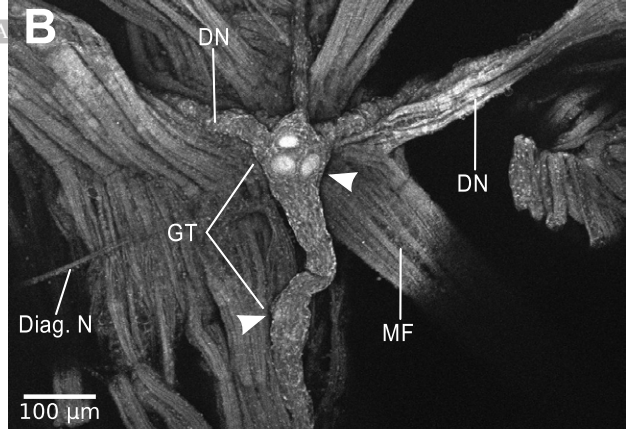
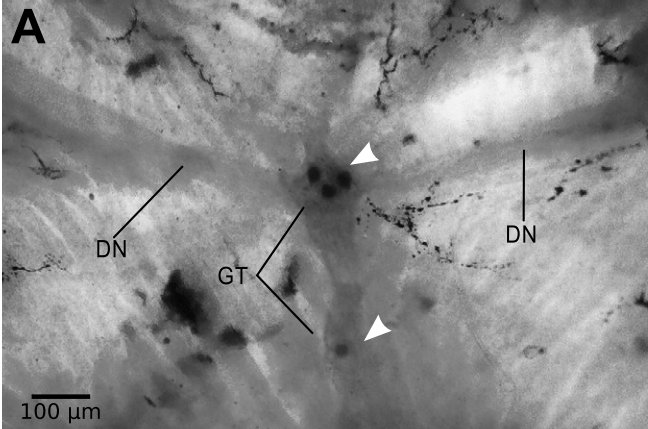
Figure 4. General organization proposed for the ganglionic trunk in *Neohelice granulata*.

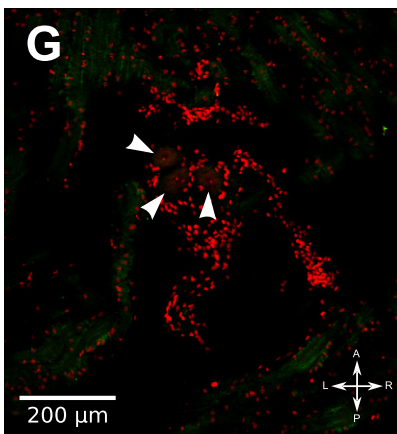
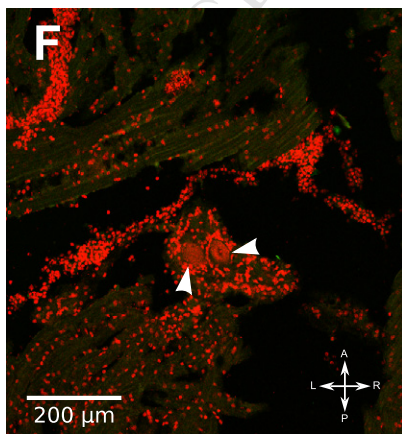
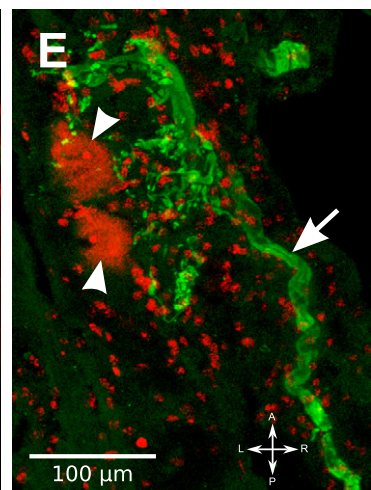
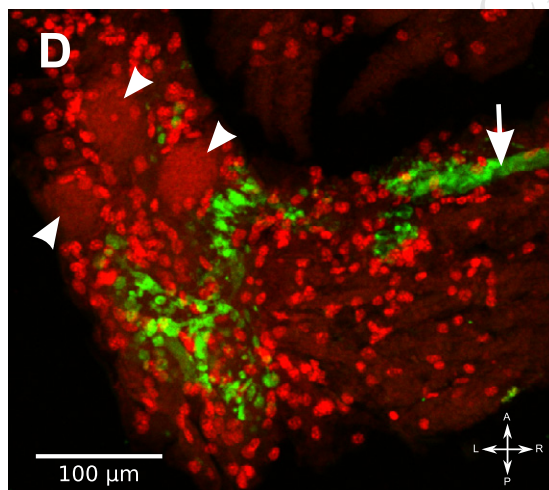
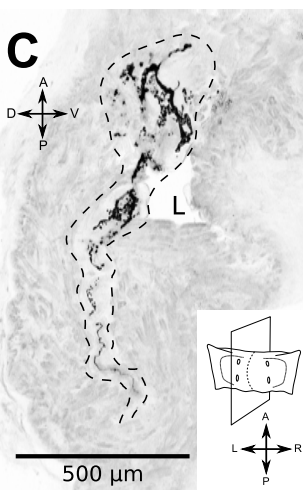
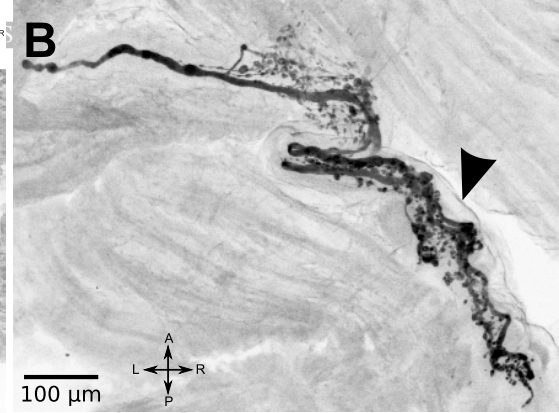
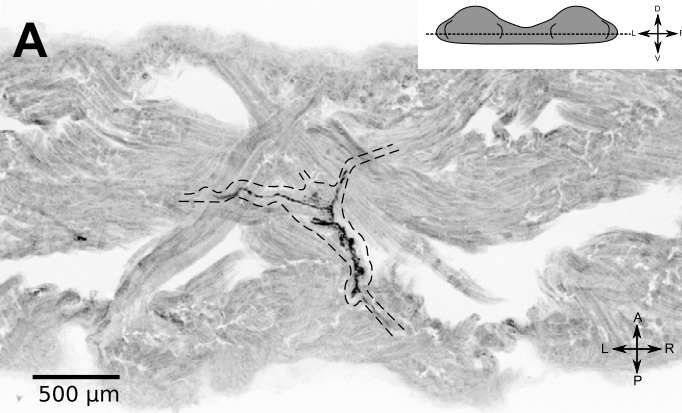
A line drawing composite from several independent observations of fresh and methylene blue staining heart preparations in which we describe the general organization of the GT. Extrinsic regulation from presumably dorsal nerves (DN) contact GT laterally from each side. The local system consists of a trunk containing large neurons and branches arising from it and distributed in the heart muscle (MF). The two branches originating from the anterior bifurcation head laterally, turn backwards, and take a circular course until they reach the posterior end of the GT. Inside the GT three of the large cell bodies are located in an anterior position of the CG and a fourth one is located more posteriorly (arrows). Thinner nerves (Diag. N) to different parts of the heart ramified diagonally from the CG.

Figure 5 supplementary material. Three dimensional view of the GT in *Neohelice*.

3D projection at maximum intensity every 10 degrees (stack of 47 images) of Fig. 2B. Three of the large cell bodies are located in an anterior position of the CG, the fourth one is located more posteriorly (arrows).









MF

DN

GT

Diag. N

A schematic diagram of a brain section, likely a coronal or sagittal slice, showing various anatomical structures. The diagram is enclosed in a dashed line. The central region contains a cluster of four small circles, with three of them labeled 'GT' (Gross Tumor) and one labeled 'Diag. N' (Diagnostic Nodule). The label 'MF' (Medial Frontal) is positioned above the central cluster, and 'DN' (Dorsal Nucleus) is positioned to the right of the central cluster. The diagram also shows a network of lines representing blood vessels or neural pathways, with shaded regions indicating specific areas of interest.